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DSK1, A Novel Kinesin-related Protein from the Diatom *Cylindrotheca fusiformis* that Is Involved in Anaphase Spindle Elongation

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Abstract. We have identified an 80-kD protein that is involved in mitotic spindle elongation in the diatom *Cylindrotheca fusiformis*. DSK1 (Diatom Spindle Kinesin 1) was isolated using a peptide antibody raised against a conserved region in the motor domain of the kinesin superfamily. By sequence homology, DSK1 belongs to the central motor family of kinesin-related proteins. Immunoblots using an antibody raised against a non-conserved region of DSK1 show that DSK1 is greatly enriched in mitotic spindle preparations. Anti-DSK1

stains the diatom central spindle with a bias toward the midzone, and staining is retained in the spindle midzone during spindle elongation in vitro. Furthermore, preincubation with anti-DSK1 blocks function in an in vitro spindle elongation assay. This inhibition of spindle elongation can be rescued by preincubating concurrently with the fusion protein against which anti-DSK1 was raised. We conclude that DSK1 is involved in spindle elongation and is likely to be responsible for pushing half-spindles apart in the spindle midzone.

Two general types of movement contribute to separating sister chromatids during anaphase, and there is evidence that both types of movement can be accomplished by multiple mechanisms. Anaphase A, or chromosome to pole movement, may be driven by molecular motors at the kinetochore (Pfarr et al., 1990; Hyman and Mitchison, 1991; Goldsmith et al., 1992; Hyman et al., 1992), by depolymerization of tubulin subunits from the plus ends of kinetochore microtubules (MTs)¹ (Gorbsky et al., 1987, 1988; Koshland et al., 1988; Coue et al., 1991; Shelden and Wadsworth, 1992; Lombillo et al., 1995), and by MT flux, the "reeling in" of MTs toward the spindle poles (Mitchison et al., 1986; Hamaguchi et al., 1987; Sawin and Mitchison, 1991; Mitchison and Salmon, 1992). Anaphase B, or spindle elongation, may be driven by forces pulling on the spindle poles from outside the mitotic spindle (Aist and Berns, 1981; Kronebusch and Borisy, 1982; Hiramoto and Nakano, 1988; Aist et al., 1991, 1993), by polymerization of interdigitating MTs (Rebhun and Palazzo, 1988; Palazzo et al., 1991), and by MT motors in the spindle midzone (McDonald et al., 1977; Leslie and Pickett-Heaps, 1983; Cande and McDonald, 1985, 1986; Masuda et al., 1990; Sullivan and Huffaker, 1992). A major challenge in studying anaphase has been the development

of model systems that isolate one particular type of anaphase movement.

Due to the exquisite structure of their mitotic spindle, diatoms have been used to study spindle morphology for over a century (Lauterborn, 1896; Pickett-Heaps and Tippet, 1978; Pickett-Heaps et al., 1984). The MTs emanating from either pole of the central spindle are approximately uniform in length and interdigitate to form a very well-defined zone of antiparallel MT overlap. The chromosomes and kinetochore MTs involved in anaphase A are spatially separated from the central spindle, forming a loose cone around the highly organized paracrystalline array of MTs that is responsible for anaphase B (McDonald et al., 1977, 1979; Pickett-Heaps and Tippet, 1978; McIntosh et al., 1979). Thus, the diatom spindle has two inherent advantages as a model system for mitotic spindle elongation: a spatial separation of anaphase A and anaphase B, and an extremely high level of MT organization in the central spindle that is responsible for anaphase B.

One of the first isolated spindles capable of mimicking anaphase movement in vitro was from the diatom *Stephanopyxis turris* (Cande and McDonald, 1985; McDonald et al., 1986). This isolated spindle model played an important role in elucidating the forces that drive anaphase B-type movement. By the addition of ATP, Cande and McDonald were able to reactivate anaphase B movement in isolated spindles lacking any cytoplasmic connections, showing that mechanical interactions in the zone of MT overlap can drive spindle elongation (Cande and McDonald, 1985, 1986). Subsequently, they were able to uncouple MT sliding from MT polymerization, demonstrating that the force

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1. Abbreviations used in this paper: BCA, bicinchoninic acid; GST, glutathione-S-transferase; KRP, kinesin-related protein; MT, microtubule.

for spindle elongation in *S. turris* is not generated by tubulin polymerization onto the ends of MTs; rather, a mechanochemical enzyme in the zone of MT overlap must be generating the forces required to push apart and separate the two half-spindles (Masuda et al., 1988).

More recently, our laboratory has turned to the pennate diatom *Cylindrotheca fusiformis* to further elucidate the proteins involved in anaphase spindle elongation (Hogan et al., 1993a). *C. fusiformis* is an ideal diatom system for the biochemical analysis of spindle proteins. Cultures can be grown to considerably higher densities than most other diatoms and can be synchronized so that up to 80% of the cells contain spindles in metaphase or early anaphase (Wein et al., 1995). These spindles are more robust than those from other organisms; they can be subjected to centrifugal or shearing forces in the course of their removal from the cell and still remain intact. Furthermore, isolated spindles or spindles within permeabilized cells can be reactivated for anaphase B movement with the addition of ATP, providing an *in vitro* assay whereby several hundred spindles may be assayed for spindle elongation in a short period of time. This model system was used to obtain both pharmacological and immunological evidence that a kinesin-related protein (KRP) is involved in anaphase spindle elongation in the diatom central spindle (Hogan et al., 1992, 1993b).

A peptide antibody raised against a conserved region in the kinesin superfamily motor domain, the anti-LAGSE antibody (Sawin et al., 1992), was found to block spindle elongation *in vitro* in *C. fusiformis* (Hogan et al., 1993b). Although this antibody is very specific for a short peptide sequence, the sequence is conserved among KRPs; because of this, anti-LAGSE recognizes multiple proteins in the diatom. To elucidate which of the anti-LAGSE-positive proteins are involved in spindle elongation, we used the antibody to screen a *C. fusiformis* cDNA expression library for KRPs, with the goal of then raising more specific antibodies for further study. One clone from this screen encodes a protein we have named DSK1 (Diatom Spindle Kinesin 1). DSK1 is recognized not only by anti-LAGSE, but also by two other antibodies that stain the diatom central spindle: the anti-HIPYR antibody, which is another kinesin peptide antibody (Sawin et al., 1992), and an mAb raised against MCAK (Wordeman and Mitchison, 1995), a centromere-associated KRP found in CHO cells. DSK1 belongs by sequence homology and domain organization to the central motor family of KRPs, which includes KIF2 (Aizawa et al., 1992) and MCAK (Wordeman and Mitchison, 1995). The members of this family appear to have very diverse functions, but none have previously been shown to play a role in anaphase B. In this paper, we present evidence that DSK1 is involved in mitotic spindle elongation.

Materials and Methods

Isolation and Cloning of the DSK1 Gene

To construct the lambda library, total RNA was prepared from mitotically synchronized *C. fusiformis* cells (Wein et al., 1995 for synchronization procedure). Cells were harvested, washed in PBS, resuspended in homogenization buffer (4.5 M guanidine isothiocyanate, 50 mM sodium citrate, pH 7,

5% β -mercaptoethanol, 0.5% sarkosyl), and placed in a bead beating chamber with an equal volume of 0.1-mm glass beads. Cells were given four 30-s pulses, and beads were removed by centrifugation. Total RNA was recovered from the homogenate using a procedure modified from March et al. (1985). Poly A⁺ RNA was isolated, and cDNA was synthesized and directionally cloned into the lambda ZAP II vector using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). 80,000 plaques were screened (Sambrook et al., 1989) on Hybond-ECL membrane (Amersham Corp., Arlington Heights, IL) with anti-LAGSE antibody at 2.4 μ g/ml (Sawin et al., 1992), and HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 0.3 μ g/ml, and then developed using ECL reagents (Amersham Corp.) according to the manufacturer's instructions. After plaque purification of LAGSE-positive clones, candidates were rescreened with the anti-HIPYR antibody at 5 μ g/ml (Sawin et al., 1992). Positive clones were then rescreened once more with the anti-MCAK mAb at 5 μ g/ml (Wordeman and Mitchison, 1995). *In vivo* excision was performed to obtain positive clones in plasmid form (Bluescript SK⁻).

Two DSK1 clones were obtained. pMAF11, which has a 1-kb insert, contains a truncated version of DSK1. pMAF24, with a 2-kb insert, contains the entire DSK1 open reading frame. Both strands of clone pMAF24 were sequenced using various subclones, deletion series (Yanisch-Perron et al., 1985), and oligonucleotide primers. Sequence analysis and comparisons were performed using the Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, WI). Coiled-coil predictions (Lupas et al., 1991) were made using the Coils Program, version 2.1 (Bioinformatics Group, Swiss Institute for Experimental Cancer Research, University of Lausanne, Switzerland), available at World Wide Web address: http://ulrec3.unil.ch/coils/COILS_doc.html.

Production of the Anti-DSK1 Antibody

A 354-bp FspI-HincII fragment of nonconserved sequence downstream of the DSK1 motor domain was cloned into the BamHI-SmaI-digested pGEX-1N vector (Smith and Johnson, 1988) (Pharmacia Biotech Inc., Piscataway, NJ) to allow the expression of a glutathione-S-transferase (GST) fusion protein (pMAF64p). Soluble fusion protein was purified on glutathione agarose beads (Smith and Johnson, 1988). Two New Zealand White rabbits were subcutaneously injected with 0.5 mg of the pMAF64p fusion protein in Freund's complete adjuvant and subsequently in Freund's incomplete adjuvant at monthly intervals. Test bleeds were taken 7 d after injections and bleeds were analyzed by Western blotting. For affinity purification, purified fusion proteins and bacterial extracts were coupled to Affigel-10 (Bio-Rad Laboratories, Hercules, CA) overnight at 4°C, and affinity purification was performed as described (Harlow and Lane, 1988) with the following modifications. Antiserum was preadsorbed on a column prepared from an extract expressing another GST fusion protein (a gift from A. Pidoux, University of California at Berkeley). The depleted serum was then passed over the pMAF64p column, and the bound antibodies were then eluted with 100 mM glycine, pH 2.5, or with 100 mM triethanolamine, pH 11.5. The peak glycine fractions were concentrated using a Centricon-10 (Amicon Corp., Danvers, MA) according to manufacturer's instructions. Protein concentrations were determined, where applicable, with the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL).

Western Blot Comparison of Whole Cell and Mitotic Spindle Extracts

For whole cell extracts, *C. fusiformis* cultures were pelleted at 2,500 g for 5 min, resuspended in PBS + 0.5% SDS, vortexed vigorously with an equal volume of 0.1-mm glass beads to break open cells, and then centrifuged briefly to remove the beads. The sample was boiled for 10 min, centrifuged briefly in a microfuge to clear the solution, and then centrifuged for 1 h at 24°C at 100,000 g. Mitotic spindles were prepared as described previously (Wein et al., 1995), with modifications as follows: after homogenization and filtration, the filtrate was diluted to a final concentration of 1.3% Triton X-100 with PMEG (50 mM Pipes, pH 7.0, 5 mM MgSO₄, 5 mM EGTA, 40 mM β -glycerophosphate, 1 μ M rac-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), 1 mM DTT, 1 mM PMSF, proteinase inhibitor cocktail) + 3% DMSO + 2% Triton X-100. The diluted filtrate was centrifuged for 10 min at 200 g to remove the diatom shells, unbroken cells, and remaining glass beads; then the supernatant was removed and centrifuged for 15 min at 3,340 g to remove remaining cellular debris. The supernatant from this spin was centrifuged through a

40% sucrose solution in PMEG at 16,500 g for 15 min to pellet spindles. Spindles were resuspended in a small volume of PMEG + 0.5% SDS and boiled for 10 min. The sample was centrifuged for 1 h at 24°C at 100,000 g in a TLA100.3 rotor in a TL100 benchtop ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Protein concentrations were determined using the BCA protein assay (Pierce Chemical Co.). Proteins (22 µg per lane) were separated on 10% polyacrylamide gels, and then transferred to nitrocellulose by semidry blotting. Blots were blocked overnight at 4°C with 5% nonfat milk in PBS + 0.2% Tween-20 and incubated with antibodies for 1 h at room temperature. Anti-DSK1 was used at 1:100, and anti-MCAK at 1:500. HRP-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:5,000 and detected using ECL reagents (Amersham Corp.) according to the manufacturer's instructions.

Immunofluorescence

Immunofluorescence was performed as described previously (Wein et al., 1995). Briefly, permeabilized cells (frozen in liquid nitrogen after the permeabilization step in the spindle isolation procedure) were centrifuged at 2,500 g for 5 min through 3 ml of PMEG + 3% DMSO onto poly-L-lysine-coated coverslips. Coverslips were fixed for 10 min in 0.1% glutaraldehyde, 0.05% paraformaldehyde in PME [50 mM Pipes, 5 mM MgSO₄, 5 mM EGTA], reduced in 1 mg/ml sodium borohydride in 50% MeOH/H₂O, and then blocked in 0.3% BSA/PBS for 20 min. Coverslips were incubated in appropriate antibodies for 1 h each: anti-DSK1 at 1:5, anti-MCAK at 1:25, monoclonal mouse anti-sea urchin α -tubulin (provided by Dr. D. Asai, Purdue University, Lafayette, IN) at a 1:500 dilution, and affinity-purified rabbit anti-soybean α -tubulin (provided by Dr. R. Cyr, Pennsylvania State University, University Park, PA) at 1:100. Coverslips were then incubated in appropriate secondary antibodies for 1 h. For double labeling, cells were incubated with both secondary antibodies simultaneously: FITC-conjugated anti-mouse (Sigma Chemical Co., St. Louis, MO) at 1:30, FITC-conjugated anti-rabbit (Cappel Research Reagents, Malvern, PA) at 1:100, Texas red-conjugated anti-mouse (Jackson ImmunoResearch Laboratories) at 1:200, and Texas red-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories) at 1:200. Coverslips were incubated in PBS + 1 µg/ml 4',6-diamidino-2-phenylindole for 5 min before mounting on slides in 90% glycerol containing 1 mg/ml 1,4-diazabicyclo[2,2,2]octane (Aldrich Chemical Co., Milwaukee, WI). Slides were observed using a Zeiss Universal Microscope with a $\times 100$ Neofluar objective (Carl Zeiss, Inc., Thornwood, NY) with epifluorescence illumination. Photographs were taken on T-MAX 100 film (Eastman Kodak Co., Rochester, NY).

In Vitro Spindle Elongation Assay and Functional Blocking with Antibodies

For the study of DSK1 distribution during spindle elongation, synchronized permeabilized cells were spun onto coverslips as described, and then incubated with 0.05 mM ATP. One coverslip was fixed every 30 s for a period of 4 min; then coverslips were all processed together as described above. For antibody inhibition studies, anti-DSK1 and fusion protein were concentrated and exchanged into PBS buffer using the Centricon-10 (Amicon Corp.). Protein concentrations were then determined using the BCA assay (Pierce Chemical Co.). All antibodies for these experiments were diluted in PBS + 0.3% BSA. Cells were spun onto coverslips as described above, and then incubated for 15 min at room temperature with antibody or buffer, as indicated. Coverslips were then rinsed in PMEG and incubated for 4 min with 1 mM ATP in PMEG or PMEG alone (for controls) before being fixed and processed. Spindle counts were made using a $\times 40$ Neofluar objective (Carl Zeiss, Inc.). Only spindles with obvious gaps were counted as elongated. The percentages of gaps on control coverslips were subtracted from the percentages on sample coverslips to get an accurate measure of the number of spindles that had elongated with each antibody or buffer solution. Each column in Fig. 6 represents a count of at least 700 spindles. Intermediate concentrations of anti-DSK1 and fusion proteins were assayed as well (data not shown), and all results were consistent with the experiments shown here.

Results

Cloning of DSK1

Two affinity-purified polyclonal antibodies raised against

conserved regions in the kinesin motor domain, termed anti-LAGSE and anti-HIPYR, stain the diatom mitotic spindle, and the anti-LAGSE antibody blocks diatom spindle elongation in vitro (Hogan et al., 1993b). The anti-LAGSE antibody was used to screen a λ -ZAP cDNA expression library constructed from mitotically synchronized *C. fusiformis* cells. Three of the positive clones were found to cross-react with both the anti-HIPYR antibody and with an mAb against MCAK (Wordeman and Mitchison, 1995) that also stains the diatom central spindle (see Fig. 4 c). Sequence analysis shows that these clones all contain cDNA encoding the same protein, which we have named DSK1 for Diatom Spindle Kinesin 1 (Fig. 1). The DSK1 open reading frame encodes a protein of predicted molecular mass of 70 kD. Southern blots confirm that DSK1 is a genomic single-copy gene (data not shown).

Analysis of the predicted amino acid sequence shows that DSK1 belongs to the family of KRPs that includes KIF2 (Aizawa et al., 1992) and MCAK (Wordeman and Mitchison, 1995). The distinctive feature of this family is that the motor domains of these proteins are centrally located (Fig. 2 A). DSK1, like KIF2 and MCAK, has two predicted coiled-coil domains, one on either side of the motor domain region. DSK1 has 47% identity to KIF2 in the motor domain (Fig. 2 B) and 37% identity throughout the whole protein. To MCAK, DSK1 has 48% identity in the motor domain and 35% identity overall. In contrast, MCAK and KIF2 are 75% identical in the motor domain and 54% identical across the entire protein.

DSK1 Is a Mitotic Spindle Protein

To obtain an antibody specific to DSK1, a unique region of sequence downstream of the motor domain was expressed as a fusion protein with GST in bacteria. Polyclonal antiserum was then raised in rabbits and affinity purified against the fusion protein.

To determine whether DSK1 is enriched in mitotic spindle extracts, we compared extracts taken from whole cells with extracts from an isolated spindle preparation that we have previously shown to be enriched by at least 15–50-fold for mitotic spindle proteins (Wein et al., 1995; changes noted in Materials and Methods). Loading approximately equal amounts of protein from each extract gives two distinct patterns of bands on a Coomassie-stained polyacrylamide gel (Fig. 3 a). On Western blots, anti-MCAK recognizes at least three bands in each of these extracts (Fig. 3 b), with bands at ~ 80 and ~ 180 kD enriched in the mitotic extract. The anti-DSK1 antibody recognizes only one band, at approximately 80 kD (Fig. 3, c and d). This band can be detected by anti-DSK1 in whole cell extracts with longer exposures of blots (Fig. 3 d), but the band is greatly enriched in the mitotic spindle extract when equal amounts of protein are compared (Fig. 3 c). This result strongly suggests that DSK1 is a component of the mitotic spindle.

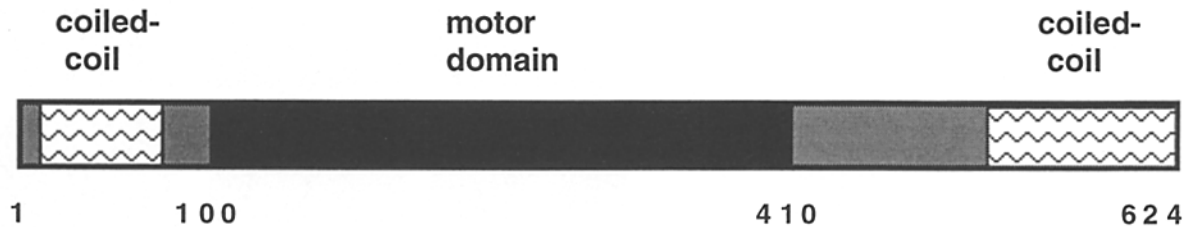
To determine the localization of DSK1, we performed immunofluorescence on permeabilized, fixed cells that had been synchronized either by using a light/dark cycle with low levels of nocodazole as previously described (Hogan et al., 1993a; Wein et al., 1995) or by using only a light/dark cycle. In the undrugged preparation, <5% of

AGATTGGAGCAAGTAGAGCATTCAATAATCGTTGGCAGACAGAAAGAAGTAGTACAATGAATCGCTGCCAATCGCCGAAAGTCAACATCCACGGTGTGGTATTACCGGACGCAAGAAAGTGTACT	120
M N A A N R R K S T S T V G I T G R K D A T	22
CGGATGAAAATAGAGCAAATGGAGAAGGAGCGTAAGGAACGTCGTAAGACTATGATGCAGCGGAAAGAAGCCCGAAAGCAAGAACATATGAAGAATATTGAAGCTGGAATCTCTGGCGAC	62
R M K I E Q M E K E R K E R R K T M M Q R K E A R K Q E H M K N I E A G N P G D	
GTTGATTTCATCGGACTCGTGGAGGAATGGCGTCGCGAACAAGAAAAAGATTGGAGACAAGTCGCCCCCATCGCTGTTTGTCTCCACGAATTCAAACATTTGTATCGCCGTTTCGAAAA	360
V D F I G L V E E W R R E Q E N K I G D K S P P S L F A S T N S N I C I A V R K	102
CGCCCCATTAGCGACAAGGAACGACAAAACTGGACCATGATCTCTGTTTCGTGCTTCCAGAACAAAGTTTGGATTCACTCTGCAAACTGAAAGTGGATGGAATTACGAAGTATTTAACT	480
R P I S D K E R Q K L D H D S V S C F Q N K V W I H S A K L K V D G I T K Y L T	142
CATAATTCCTTTTCAGCTCGATCATACCTTTGGAGAAGATTCTACGACTGAACAAATCTATCTCGCTACAACCTCTGCCACTGCTCGACCATGTCGTATCTACCCAGGGCAGAGCGACTGTC	600
H N S F Q L D H T F G E D S T T E E Q I Y L A T T L P L V D H V V S T G C Q G R A T V	182
TTTTGCTATGGACAAACTGGTAGTGGAAGACATACGATGAACGGAATTTCAACAAATCTTGGCATACGATTTATACGGACAACCTTCGAGAATCACCACGACCTGGAAATCACGGTC	720
F C Y G Q T G S G K A T Y T M N G I Q Q I L A Y L A T T L P L V D H V V S T G C Q G R A T V	222
GCCTTTTTGAATTGTACTCTGGCAATGTGCTGGACCTCTTCGATGGATGCCAACGCTGCAAACTGCTAGAAGACGGCAATGGTGAAGTTAATATTACTGGTTTGAGAGAAGTTCCGGCC	840
A F F E L Y S G N V L D L L H G C Q R C K L L E D G N G E V N I T G L R E V P A	262
CCAACCCAGAAAGCCTTTCTGCAAGTGATCGAAGAAGGCCACAGTCTTCGAACAACTCAGAAAACCGAAGCAACAGATGCCTCTTCGAGGTCTCATGCCATTTGCCAAGTCTTTCTCCGA	960
P T P E A F L Q V I E E G H S L R T T Q K T E A N D A S S R S H A I C Q V F L R	302
GACTATGGCGGGAACCTCAGAGGAAGCTGGGTTTGGTAGATTGGCTGGTAGTGAACGCTGGATCGGATACAAAACAGCACAAATTCACAACGAAGAAGCGAATCTGCTGATATCAATACA	1080
D Y G N L R G K L G L V D L A G S E R G S G G A T K Q H N S Q R R T E S A D I N T	342
TCTCTACTGGCATTGAAGGAGTGCAATTCGTGCTTTGGGACAGAACTCTGCCACGTAACCCCTACCGTGGATCCAAATTGACATCTTAAGGATTGTTCTCACCTGACTCCAAGACG	1200
S L L A L L K E C I R A L G Q K S A H V P Y R G S K L T L I L K D C F S P D S K T	382
ACAATGGTGGCAGCTGTCTCGCCTGGGCTCTCGAGCTGATCACTCTTTGAATACTCTGCGATATGCTGATCGAATCAAGGAGCAAAGGTTTCGAGTAATGGACAAAGAGGAAAGGCG	1320
T M V A T V S P G A S A A D H S L N T L R Y A D R I K E Q R V S S N G Q R G K A	422
GCAAAAGCCAGCAACCGAGAATAATGCTTCGAAAGAAGCGCTTGTGCGCAATTCGAGCTGCCACAGAGCAAGCAGATGACCAACACAGCGCGTTTGTTCAGAAGATGCTGGCCGAACAC	1440
A K A S N R E I M P S K E R L M R I A A A T E Q A D D Q H S A F V Q K M L A E H	462
GATCAGGTACAGGCTGATGCCAATGATTATGCTTTACAGAGCAAGTAGACGAAGAAGAGCAGACGATGAAGAAGGTGACTATGAAGAAGAGTCTGAAGATCTCGACTATGAAGACTCG	1560
D Q V Q A D A N D Y A F T E Q V D E E E A D D E E G D Y E E E S E D L D Y E D S	502
GAAGGACAGGACTATGAAGAAGCTGTGGAATCGCAGTACGACCATTCACAAGAAGCCAGGAGGGTGAGGAAGAGTCTCGCAAGTGTTCAGACCGTTTCGAGCTGGAGGAAGCGCTG	1680
E G Q D Y E E A V E S Q Y D H S Q E A Q E G E E L R R T V Q A V F E L E A L G	542
CTGAATCAGCATATGAGCAATATTTCAGGCTAACGCCGAATTTGTGACGAAGAAGAGCTGCTGCAATCAGTTCAAAGCCGAGGCTTATCGGAAGACGAGATGCACAACTACGCAATC	1800
L N Q H M S N I Q A N A E M L T A H Q E G K L L Q S V Q A G G L S E D E M H N Y A I	582
CAACTAGCTGAGTTTCTGGATAAGAAGGAATCGTTGATTACAACTGCAATCAAAGCTTGACGAATTCCAGGAGCAGCTAGCAAGGAGCAGGAGTTGGCAGCGCAAGTCCAGTTGACA	1920
Q L A T T E A F L D K K E S L I Y K L Q S K L D E F Q E Q L A R E Q E L A R Q V Q L T	622
CAATATTGAATTCAATTTTGTATCGTCGCTTGTCAAAAGTAGGCAAAAGGTATCTCTGGCCCCCTCGTC	1988
O Y *	624

the cells contain spindles, but it is important to use for localization studies because cells at all stages of the cell cycle are present. Treatment with nocodazole greatly slows progression through mitosis, allowing for the capture of up to 80% of cells in early anaphase by using a permeabilization step that halts anaphase movement completely. Nocodazole causes the depolymerization of most cytoplasmic MTs and, due to the absence of astral MTs, spindles within these drugged cells reposition perpendicular to their physiological orientation (Fig. 4, *b*, *e*, and *h*). Spindle morphology is slightly different as well—spindles in the drugged cells tend to be slightly longer and thinner than those in undrugged cells. However, both types of spindles still have the well-defined zones of MT overlap necessary for spindle elongation (Bove, C., and W.Z. Cande, unpublished observations).

During spindle elongation, spindles form a gap at their midzone as the two half-spindles slide apart and separate (Fig. 4 *k*). In spindles that have fully elongated *in vitro*, DSK1 staining has almost completely disappeared from the mitotic spindle (Fig. 4 *l*). This decrease in staining after spindle elongation was also seen using the anti-MCAK antibody (data not shown). Extremely low levels of staining can be detected throughout some late anaphase or telophase spindles, but this level of staining represents a considerable decrease in DSK1 localization compared to metaphase or early anaphase spindles.

A



B

DSK1	NICIAVRKRP	ISDKERQKLD	HDSVSC.FQN	KVWIHS AKLK	VDGITKYLTH	143
KIF2	RICVCVRKRP	LNKKETQMKD	LDVITIPSKD	VVMVHEPKQK	VD.LTRYLEN	243
MCAK	RICVCVRKRP	LNKQELAKKE	IDVISVPSKC	LLFVHEPKLK	VD.LTKYLEN	290
DSK1	NSFQLDHTFG	EDSTTEQIYL	ATTLPVLDHV	VSTQGRATVF	CYGQTGSGKT	193
KIF2	QTFRFDYAFD	DSAPNEMVYR	FTARPLVETI	F.ERGMATCF	AYGQTGSGKT	292
MCAK	QAFCFDFGFD	ETASNEVVYR	FTARPLVQTI	F.EGGKATCF	GYGQTGSGKT	339
DSK1	YTMNG.....IQQILAYDLY	GQLAEHTDDL	EITVAFFELY	228
KIF2	HTMGDFSGK	NQDCSKGIYA	LAARDVFLML	KKPNYKKLEL	QVYATFFEIY	341
MCAK	HTMGDCLVN	LRIHLKGILCFLLK	SQPRYRNLM	EVYVTFFEIY	383
DSK1	SGNVLDLLHG	CQRCKLEDG	NGEVNITGLR	EVPAPTPEAF	LQVIEEGHSL	278
KIF2	SGKVFDDLNR	KTCLRVLLEDG	KQQVQVVLQ	EREVKCEDV	LKLIDIGNSC	392
MCAK	NGKVFDDLNK	KAKLRVLEDS	KQQVQVVLQ	EYLVNCADDV	IKMLNMGSA	433
DSK1	RTTQKTEAND	ASSRSHAICQ	VFLRDYGGNL	RGKLGVLDA	GSEGRSDTKQ	328
KIF2	RTSGQTSANA	HSSRSHAVFQ	IILRR.KGKL	HGKFSLIDLA	GNERGADTSS	440
MCAK	RTSGQTFANS	NSSRSHACFQ	ILLRA.KGRL	HGKFSLVDLA	GNERGADTSS	482
DSK1	HNSQRRTESA	DINTSLLALK	ECIRALGQKS	AHVPYRGSKL	TLILKDCF.S	377
KIF2	ADRQTRLEGA	EINKSLLRLK	ECIRALGRNK	PHTPFRASKL	TQVLRDSFIG	491
MCAK	ADRQTRMEGA	EINKSLLALK	ECIRALGQNK	AHTPFRESKL	TQVLRDSFIG	532
DSK1	PDSKTTMVAT	VSPGASAADH	SLNTLRYADR	IKE	410	
KIF2	ENSRTCMIAT	ISPGMASCEN	TLNTLRYANR	VKE	524	
MCAK	ENSRTCMIAM	ISPGISSCEY	SLNTLRYADR	VKE	565	

Figure 2. DSK1 belongs to the central motor domain family of KRPs. (A) Domain organization of DSK1. Putative motor domain and predicted coiled-coil regions are indicated. (B) Comparison of amino acid sequences in the DSK1, KIF2 (Aizawa et al., 1992), and MCAK (Wordeman and Mitchison, 1995) motor domain regions. DSK1 shares 47% identity with KIF2 in the motor domain and 48% identity with MCAK. In contrast, KIF2 and MCAK share 75% motor domain identity.

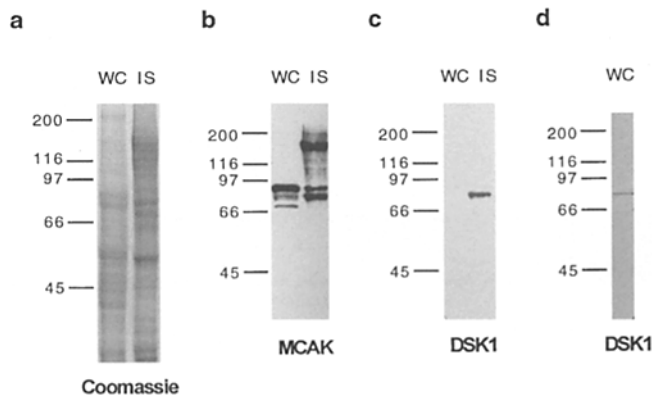


Figure 3. DSK1 is enriched in mitotic spindle preparations. Equal amounts of protein were loaded in each lane. WC, whole cell extract. IS, isolated spindle preparation extract. (a) Coomassie-stained polyacrylamide gel. (b) Immunoblot probed with anti-MCAK. (c) Immunoblot probed with anti-DSK1. (d) A long exposure of a whole cell extract probed with anti-DSK1.

addition of low levels of ATP (0.05 mM). At this ATP concentration, spindle elongation proceeds at a slow rate (Hogan et al., 1992), allowing us to monitor spindles at various stages during elongation. Fig. 5 shows several cells that were fixed as the half-spindles began to separate. Tubulin staining (*bottom row*) reveals the newly formed gap between the two half-spindles. The top row shows the same cells stained with anti-DSK1. Consistently, DSK1 staining is concentrated in the forming gap, suggesting that DSK1 may be part of a matrix whose presence in the midzone is defined by more than simply the presence of antiparallel MT arrays. Alternatively, DSK1 may be concentrated toward the ends of the few remaining antiparallel MTs still interacting in the midzone. We are currently investigating these two possibilities. Subsequent to this concentration of DSK1 in the midzone, which occurs from 1 to 2.5 min after the addition of ATP, DSK1 is found at much lower levels in the spindle, and staining looks similar to that shown in Fig. 4 *l*.

DSK1 Is Directly Involved in Spindle Elongation

We tested several antibodies for inhibition of spindle elongation, including anti-LAGSE, anti-MCAK, anti-DSK1, and an antibody against tubulin. To test whether an antibody perturbs spindle elongation in our assay, we preincubate permeabilized cells with the antibody for 15 min, wash out the antibody, add 1 mM ATP for 4 min to reactivate spindle elongation, and then fix and process for immunofluorescence. The presence of a bend or a gap in its midzone indicates that a spindle has undergone elongation (Hogan et al., 1992). Fig. 6 *a* shows a control field of permeabilized cells containing spindles that have elongated *in vitro* after the addition of ATP. Note the gaps that have formed between half-spindles after they slide apart and separate; some of the spindles also exhibit slight bends or twists. Fig. 6 *b* shows a field of spindles that have been preincubated with anti-DSK1 at 120 μ g/ml. Few spindles now exhibit gaps or bends, indicating that anti-DSK1 has inhibited most of the spindles from elongating.

Fig. 7 shows the results from three sets of antibody inhi-

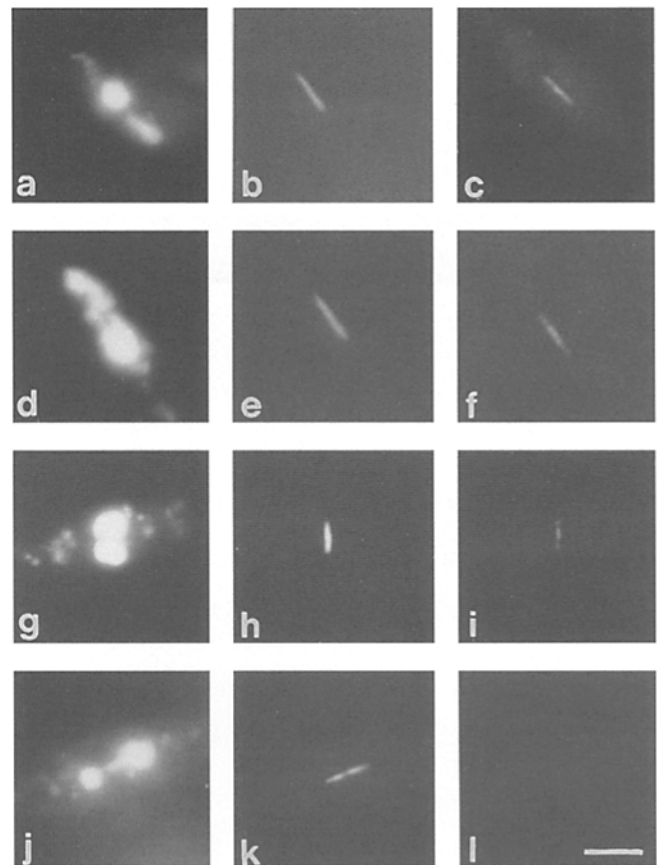


Figure 4. DSK1 localizes to the mitotic spindle. (a, d, g, and j) Cells were stained with 4',6-diamidino-2-phenylindole to visualize DNA. (b, e, h, and k) Staining of the mitotic spindle with anti- α -tubulin antibody, visualized with Texas red-conjugated secondary antibody. (c) Immunolocalization of MCAK, visualized with FITC-conjugated secondary antibody. (f) Immunolocalization of DSK1, also visualized with FITC-conjugated secondary antibody. (i) DSK1 in an undrugged cell, synchronized with a light/dark cycle only (see text). (l) DSK1 staining in a spindle that has completely elongated after the addition of 1 mM ATP for 4 min. Bar, 6 μ m.

bition experiments. The anti-LAGSE antibody has been previously shown to inhibit spindle elongation (Hogan et al., 1993b) and provides a positive control for our assay (Fig. 7 *A*). The anti-MCAK antibody, which recognizes a subset of the anti-LAGSE proteins, also inhibits spindle elongation (Fig. 7 *A*). Anti-DSK1 recognizes only one band on a Western blot; however, it inhibits spindle elongation to a greater extent than do either the anti-LAGSE or anti-MCAK antibodies used at similar concentrations (Fig. 7 *A*). The degree of inhibition is dependent on the concentration of anti-DSK1 (Fig. 7 *B*). Boiled anti-DSK1 does not inhibit spindle elongation, indicating that active antibody is needed for inhibition (Fig. 7 *B*). An antibody directed against α -tubulin does not inhibit spindle elongation, even when spindles are preincubated with antibody at very high levels (Fig. 7 *B*), showing that the binding of an antibody to the mitotic spindle and its midzone is not sufficient to inhibit spindle elongation.

To investigate whether inhibition of spindle elongation by the anti-DSK1 antibody is due to specific binding be-

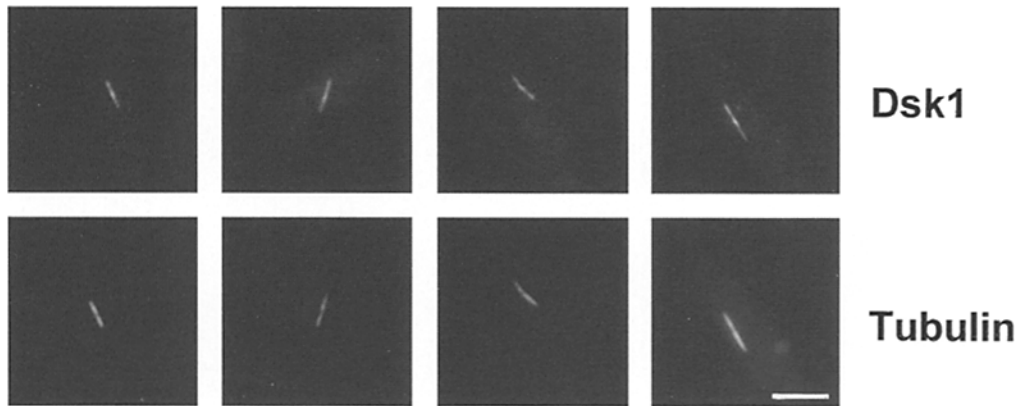


Figure 5. DSK1 is concentrated in the spindle midzone during *in vitro* spindle elongation. Spindles were fixed after 1–3 min in 0.05 mM ATP, before spindle elongation was complete. (*Top row*) Immunolocalization of DSK1, visualized with FITC-conjugated secondary antibody. (*Bottom row*) Staining of the spindles with anti- α -tubulin antibody, visualized with Texas red-conjugated secondary antibody. Note that DSK1 staining is concentrated at the same location as the forming gap between the two half-spindles. Bar, 6 μ m.

tween the antibody and its ligand, we tested whether the fusion protein against which anti-DSK1 was raised could rescue the antibody's inhibitory effect. Indeed, as shown in Fig. 7 C, low amounts of fusion protein partially rescue spindle elongation, while higher amounts of fusion protein completely titrate away the anti-DSK1 inhibition, bringing the percentage of spindles elongated back up to control levels. This experiment demonstrates that the anti-DSK1 antibody inhibits spindle function by specifically binding to endogenous DSK1 protein.

Discussion

DSK1 Is a Novel Member of the Central Motor Domain Family of Kinesins

By motor domain sequence homology and domain organization, DSK1 is most similar to the central motor family of kinesins, distinguished by the positioning of their motor domains near the center of the protein and by their two predicted regions of coiled-coils, one on either side of the motor domain.

Two distinct classes are emerging within the central motor domain family. One class consists of KIF2, which was

isolated by PCR from murine brain cDNA and appears to be involved in axonal organelle transport (Aizawa et al., 1992; Noda et al., 1995), and XKIF2 from *Xenopus* (GenBank accession No. U36486 provided by C. Walczak and T. Mitchison), which is 90% identical overall to KIF2 and is likely to be a true functional homologue of KIF2. MCAK, isolated from a CHO cDNA expression library with the anti-LAGSE and anti-HIPYR antibodies, is a mitotic centromere-associated protein (Wordeman and Mitchison, 1995) that establishes another class within this family. XKCM1 from *Xenopus*, which is highly homologous to MCAK and exhibits a similar pattern of localization, appears to play a role in MT dynamics (Walczak et al., 1996). rKRP2, isolated from rat testes, is an additional KRP in this class that was found to be expressed in the seminiferous epithelium, a tissue rich in meiotically dividing cells (Sperry and Zhao, 1996). The characterizations of these central motor family members show that, despite their sequence and domain similarity, individual members of one KRP family may perform vastly different functions.

We demonstrate here that DSK1 is involved in anaphase spindle elongation, but it is still unclear whether DSK1 is a true member of either of the two previously defined classes within the central motor domain family. While MCAK and KIF2, which are now considered to be-

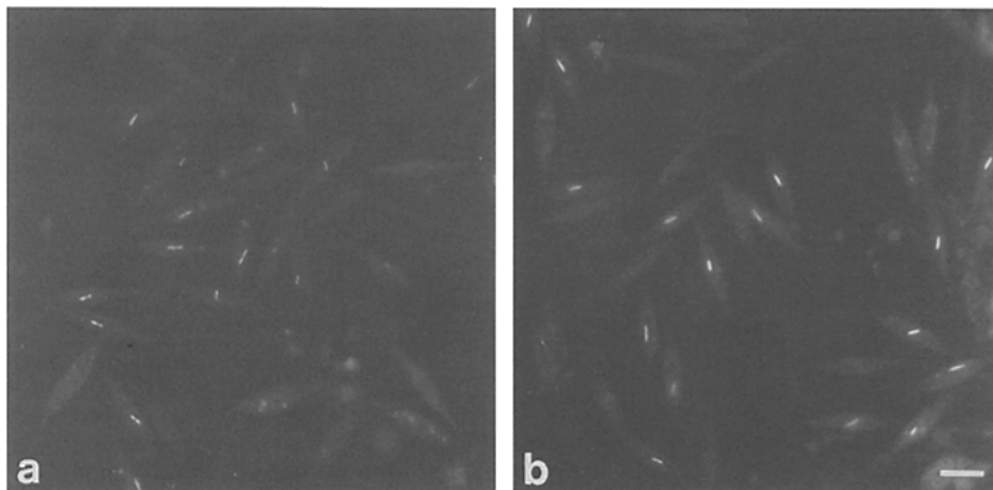


Figure 6. Anti-DSK1 inhibits spindle elongation. (*a*) Permeabilized cells containing spindles that have elongated *in vitro* after the addition of 1 mM ATP. (*b*) Cells that have been preincubated with anti-DSK1 and rinsed before the addition of ATP. Bar, 15 μ m.

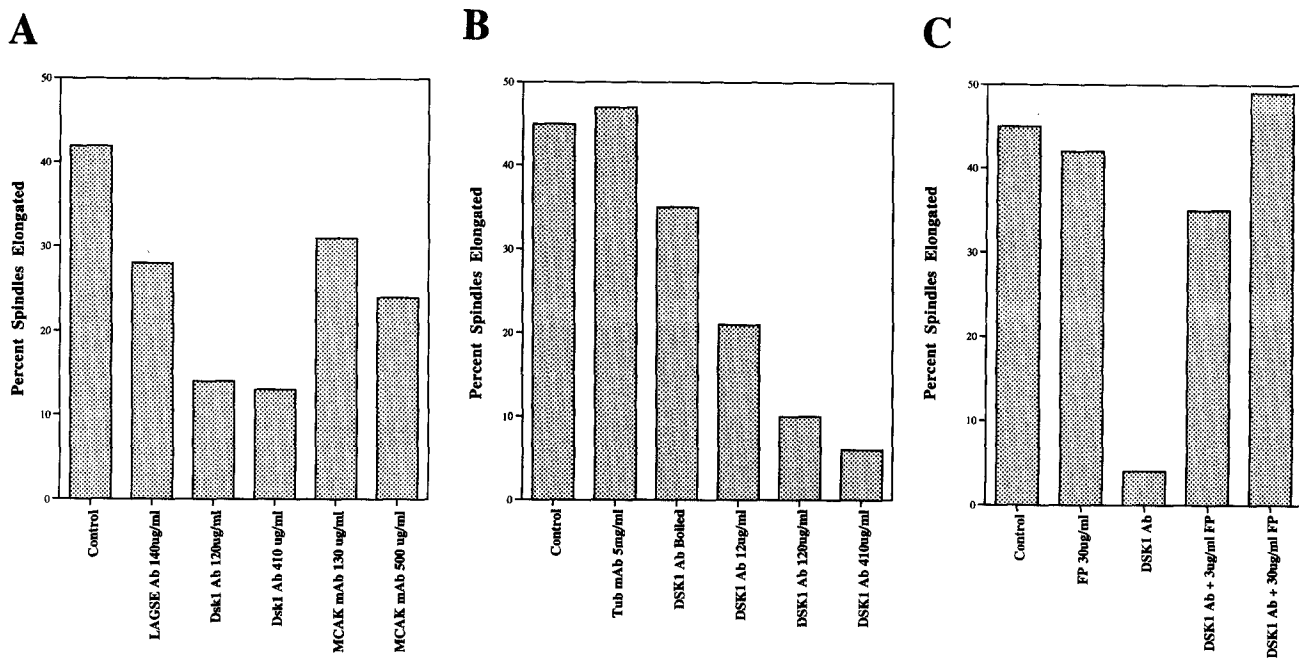


Figure 7. Results of three antibody inhibition experiments. Permeabilized cells were preincubated for 15 min with each solution and rinsed before the addition of either PMEG or PMEG + ATP for 4 min. Only spindles with obvious gaps were counted as elongated. For each preincubation solution, the percentage of gaps on coverslips incubated with PMEG alone was subtracted from the percentage on coverslips incubated with PMEG + ATP. Each column in this figure represents a count of at least 700 spindles. *FP*, fusion protein pMAF64p, against which anti-DSK1 was raised. The boiled anti-DSK1 concentration in *B* was 410 μ g/ml. The anti-DSK1 concentration in *C* was 120 μ g/ml.

long to two distinct classes, are ~75% identical to each other in the motor domain, DSK1 shares <50% identity with either. These differences suggest that, while DSK1 is closest to the central motor family in sequence (its identity to the motor domains in other KRPs is 35% or less), it may represent a third class within the central motor family or may even be a founding member of a new central motor KRP family. Alternatively, since its domain organization is similar to the other central motor KRPs, DSK1's diversity in sequence homology may simply reflect the fact that the diatom is so evolutionarily divergent from the vertebrates in which these other proteins were found.

DSK1 Is Involved in Anaphase Spindle Elongation

The anti-LAGSE, anti-HIPYR, and anti-MCAK antibodies all label the diatom central spindle, with preferential staining toward the spindle midzone, precisely where a spindle elongation motor would function if it were pushing the half-spindles apart. Anti-LAGSE and anti-MCAK also inhibit spindle elongation *in vitro*. DSK1 now seems the most likely target for these antibodies in the diatom spindle, but although all these antibodies cross-react with colonies of bacteria expressing DSK1 fusion protein, all of them also recognize multiple proteins in diatom extracts, suggesting that they may be recognizing other spindle proteins besides DSK1.

The affinity-purified anti-DSK1 antibody recognizes only one band in diatom extracts, and this band is greatly enriched in mitotic spindle preparations. Anti-DSK1 localizes to the diatom mitotic spindle, with a bias toward the midzone, and inhibits spindle elongation to a greater ex-

tent than do either the anti-LAGSE or anti-MCAK antibodies at similar concentrations. The anti-DSK1 antibody may be a more effective inhibitor of spindle elongation because it recognizes only DSK1 protein, while anti-LAGSE and anti-MCAK cross-react with nonmitotic proteins. These different antibody inhibitions may also reflect their different affinities for DSK1 or may be a result of their binding to different antigenic sites. In support of the latter possibility, the anti-HIPYR antibody also cross-reacts with DSK1 but does not inhibit spindle elongation (Hogan et al., 1993b). A similar effect has been demonstrated using mAbs that all bind efficiently to sea urchin egg kinesin yet, due to their spatially distinct binding sites, have differing effects on its translocation of MTs (Ingold et al., 1988).

The mechanism of DSK1 action in the spindle midzone during anaphase has yet to be elucidated. A bacterially expressed portion of the DSK1 motor domain exhibits some kinesin-like properties, including ATPase activity and the ability to bind MTs (data not shown), but attempts to demonstrate motility and directionality of MT gliding have not yet been successful. KIF2 is the only member of this family whose directionality has been established (Noda et al., 1995). It shows plus-end-directed movement, consistent with the expected behavior of a hypothetical spindle elongation motor. Regardless of whether a molecular motor directly cross-links antiparallel MTs in the spindle midzone or whether it is part of a spindle matrix that interacts with MTs, the spindle elongation motor must be plus-end directed for it to push the half-spindles apart and to cause spindle elongation (Cande and Hogan, 1989).

While anti-DSK1 may inhibit spindle elongation by binding directly to the motor that pushes half-spindles apart, it

may also be sterically hindering spindle elongation because of its proximity to the true anaphase B motor. DSK1 may not itself be the spindle elongation motor, but rather function to keep the motor complex in the midzone. Alternatively, it may be involved in arranging and maintaining the high level of antiparallel MT organization in the midzone of the diatom central spindle. Another central motor domain KRP, XKCM1, plays a role in MT dynamics in vitro (Walczak et al., 1996), but we have no evidence that DSK1 plays such a role in the diatom; depolymerization does not appear to occur from the plus ends of the central spindle MTs during in vitro spindle elongation (Masuda et al., 1988, 1990; Hogan et al., 1992).

At this time, we cannot rule out an additional role for DSK1 in mitotic spindle assembly. In any case, functional blocking by anti-DSK1 in our in vitro spindle elongation assay strongly implicates DSK1 in *C. fusiformis* anaphase B movement. To further address the mechanism of DSK1 activity, we are currently attempting to purify the native DSK1 protein complex to assay its MT binding and motility characteristics.

Kinesin-related Proteins Involved in Spindle Elongation

With the addition of DSK1, proteins belonging to three KRP families have now been implicated in spindle elongation during anaphase. MKLP1 from mammalian cells (Nislow et al., 1992) is a strong candidate for a spindle elongation motor. This protein localizes to the spindle midzone of PtK1 cells, and a bacterially expressed motor domain causes antiparallel MTs to slide apart, a characteristic consistent with a conjectured spindle elongation motor. Microinjection of mAb that recognizes MKLP1 leads to mitotic arrest at metaphase (Nislow et al., 1990) and a disruption of the antigen's localization at the midzone. Mutants in members of another KRP family that includes bimC from *Aspergillus nidulans* (Enos and Morris, 1990), cut7 from *Schizosaccharomyces pombe* (Hagan and Yanagida, 1990, 1992), and Cin8p and Kip1p from *Saccharomyces cerevisiae* (Hoyt et al., 1992; Roof et al., 1992) all fail to separate their spindle pole bodies to form a bipolar spindle, a phenotype consistent with these KRPs playing a role in pushing half-spindles apart. After their involvement in the formation of the growing spindle, these proteins could then continue the same type of movement to cause spindle elongation during anaphase. Cin8p and Kip1p have both been implicated in anaphase B; in the budding yeast, spindle elongation is accomplished by the combined actions of dynein and both of these KRPs (Saunders et al., 1995).

DSK1, from *C. fusiformis*, defines a new class of kinesin that is involved in spindle elongation. As members of three KRP families have now been implicated in the process, it seems likely that, while the general mechanisms of spindle elongation remain the same from organism to organism, different KRPs may have evolved in divergent organisms to accomplish these same tasks. It also appears that spindle elongation is performed by multiple redundant proteins within a given cell to ensure the fidelity of chromosome separation and distribution. We are currently searching for proteins from other KRP families to ask whether they too play a role in spindle elongation in the diatom.

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